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RESEARCH PAPER

A new dual-specific incompatibility allele revealed by absence of glycosylation in the conserved C2 site of a *Solanum chacoense* S-RNase

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Abstract

The stylar determinant of gametophytic self-incompatibility (GSI) in Solanaceae, Rosaceae, and Plantaginaceae is an S-RNase encoded by a multiallelic *S*-locus. The primary structure of S-RNases shows five conserved (C) and two hypervariable (HV) regions, the latter forming a domain implicated in *S*-haplotype-specific recognition of the pollen determinant to SI. All S-RNases are glycosylated at a conserved site in the C2 region, although previous studies have shown that N-linked glycans at this position are not required for *S*-haplotype-specific recognition and pollen rejection. Here the incompatibility phenotype of three constructs derived from an originally monoglycosylated S_{11}-RNase of *Solanum chacoense*, that were designed to explore the role of the HV domain in determining pollen recognition and the role of the N-linked glycan in the C2 region, is reported. In one series of experiments, a second glycosylation site was introduced in the HVa region to test for inhibition of pollen-specific recognition. This modification does not impede pollen rejection, although analysis shows incomplete glycosylation at the new site in the HVa region. A second construct, designed to permit complete glycosylation at the HVa site by suppression of the conserved site in the C2 region, did increase the degree of site occupancy, but, again, glycosylation was incomplete. Plants expressing this construct rejected S_{11} pollen and, surprisingly, also rejected S_{13} pollen, thus displaying an unusual dual specificity phenotype. This construct differs from the first by the absence of the conserved C2 glycosylation site, and thus the dual specificity is observed only in the absence of the C2 glycan. A third construct, completely lacking glycosylation sites, conferred an ability to reject only S_{11} pollen, disproving the hypothesis that lack of a conserved glycan would confer a universal pollen rejection phenotype to the plant.

Key words: Allelic recognition, gametophytic self-incompatibility, glycosylation, site-directed mutagenesis, S-RNase, *Solanum chacoense.*

Introduction

Self-incompatibility (SI) is a genetic mechanism widespread among flowering plants that allows the pistil of a flower to discriminate between genetically related (self) and unrelated (non-self) pollen (de Nettancourt, 2001). In the Solanaceae, Rosaceae, and Plantaginaceae, the pollen phenotype is determined by its haploid genotype (gametophytic SI, or GSI), and is controlled by a multigenic *S*-locus, inherited as a single segregating unit, which contains polymorphic male and female determinants to SI. Variants of the *S*-locus are termed *S*-haplotypes, whereas variants in the polymorphic genes of the *S*-locus are called alleles (McCubbin and Kao, 2000). In the families mentioned above, the pistil-expressed *S*-gene product is an extremely polymorphic glycoprotein with RNase activity termed S-RNase (McClure *et al*., 1989). The catalytic activity of S-RNases is essential for pollen rejection (Huang *et al*., 1994; Royo *et al*., 1994) and this cytotoxic activity must thus be exerted inside the incompatible pollen tubes. However, since S-RNases have been shown by immunolocalization to

enter pollen tubes in a non-*S*-haplotype-specific manner (Luu *et al*., 2000; Goldraij *et al*., 2006), at least part of the SI mechanism must involve the ability of compatible pollen tubes to block the RNase activity of any non-self S-RNase. This blockage could occur by inhibition of the RNase activity (Kao and McCubbin, 1996; Luu *et al*., 2001; Sims and Ordanic, 2001), by degradation of the S-RNase (Qiao *et al*., 2004; Hua and Kao, 2006, 2008; Hua *et al*., 2008; Meng *et al*., 2011*a*), or by blocking access to the RNA substrates in the pollen tube cytosol (McClure, 2008; McClure *et al*., 2011).

Sequence comparisons of S-RNases in the Solanaceae have revealed a pattern of highly five conserved (C1–C5) and two hypervariable (HV) regions (Parry, 1997). The conserved regions C1, C4, and C5 appear to be involved in stabilizing the three-dimensional structure of the S-RNase (Ioerger *et al*., 1991; Qin *et al*., 2005), whereas the conserved regions C2 and C3 each contain a histidine residue essential for RNase activity (Green, 1994). The two HV regions (HVa and HVb) are thought to play a key role in *S*-haplotype specificity (Kao and McCubbin, 1996; Matton *et al*., 1997, 1999; Zurek *et al*., 1997), although other motifs of the protein can contribute to allele specificity (Verica *et al*., 1998). In the Rosaceae, domains outside the HV region have been shown to be key in determining allelic specificity, as S_6- and S_{24}-RNases in *Prunus* (Rosaceae) are identical in their HV region yet have distinct incompatibility phenotypes (Wunsch and Hormaza, 2004), and similar observations have been made in *Pyrus* (Zisovich *et al*., 2004). The HV region thus appears not to be the sole determinant for specific pollen rejection.

Curiously, all S-RNases known to date possess potential N-glycosylation sites, the role of which, if any, is still unknown (Meng *et al*., 2011*b*). For example, S-RNases from *Nicotiana alata* have from one to five sites (Oxley *et al*., 1996, 1998), while those from *Solanum chacoense* contain from one to four sites (Qin *et al*., 2001; Liu *et al*., 2008). There can be differences in the type and structure of the side chains of sugars, termed microheterogeneity, attached to the same S-RNase (Woodward *et al*., 1992; Oxley and Bacic, 1995) and, in S-RNases with multiple sites, some may remain unoccupied (Parry, 1997). In the Solanaceae, a single glycosylation site, at the N-terminus of the C2 region, is conserved in practically all cases (Singh and Kao, 1992) [with the exception of the S_1-RNase from *Petunia hybrida* whose single glycosylation site is located near the C-terminus of the protein (Clark *et al*., 1990)]. In the Rosaceae, the conserved glycosylation site is located in the fourth conserved region 4 (RC4) (Ishimizu *et al*., 1998).

The role of the sugar moieties may be that generally ascribed to secreted proteins, such as facilitation of protein secretion or increased solubility in the extracellular matrix (McClure, 2009). However, enzymatic removal of the glycan side chains has no effect on RNase activity of the protein (Broothaerts *et al*., 1991), and removal of the C2-glycosylation site of the *P. hybrida* S_3-RNase by site-directed mutagenesis did not alter the ability fully to reject S_3 pollen (Karunanandaa *et al*., 1994). Alternatively, it is possible that the sugar groups may play a more vital role in the SI response. In the case of the *Petunia* S_3-RNase without a glycosylation site, the rejection of other pollen haplotypes was not investigated, and it has been suggested that these transgenic plants might have acquired the ability to reject any pollen type (Oxley *et al*., 1996). This possibility is intriguing as the *S*-locus product expressed in pollen (pollen-*S* determinant) has been identified as an F-box protein (Lai *et al*., 2002), and N-linked glycans can be a specific target of F-box proteins (Yoshida, 2007). It is also an interesting coincidence that *S-like* RNases, not involved in SI, are not glycosylated (Green, 1994).

In order to explore the role of glycosylation in the SI response of *S. chacoense*, two constructs, both with a new N-glycosylation site inside the HVa region of the S_{11}-RNase, were generated. In one, the original glycosylation site in the C2 region (Saba-El-Leil *et al*., 1994) was maintained, whereas in the other it was removed. Interestingly, the monoglycosylated transgenics harbouring only a glycosylation site in the HVa region were found to reject S_{13} pollen in addition to S_{11} pollen. Western analyses revealed the presence of two size classes of protein, suggesting that the non-glycosylated form may reject the S_{11} pollen while the glycosylated form may reject S_{13} pollen. In order to confirm that the non-glycosylated form would reject S_{11} pollen, the conserved glycosylation site in the C2 region of the native S_{11}-RNase was eliminated, validating its ability to reject S_{11} pollen. This construct also allowed a test of the hypothesis that an S-RNase lacking N-linked glycans might show a universal pollen rejection phenotype.

Materials and methods

Plant material

The self-incompatible diploid genotypes ($2n=2x=24$) of *S. chacoense* used in the present study include the previously described genetic lines PI 230582 ($S_{13}S_{14}$) (Rivard *et al*., 1989), L25 ($S_{11}S_{12}$) (Qin *et al*., 2001), G4 ($S_{12}S_{14}$) (Van Sint Jan *et al*., 1996), a tetraploid line ($S_{11}S_{11}S_{13}S_{13}$) (Luu *et al*., 2001), and PI458312 ($S_{15}S_{16}$) (Liu *et al*., 2009).

Mutagenesis of the S_{11}-RNase glycosylation sites and plant transformation

A previously described construct (Qin *et al*., 2005) containing the wild-type S_{11}-RNase (hereafter identified as Glc^{C2} to indicate that it contains only the endogenous N-glycosylation site located in the C2 conserved region) under control of the chitinase promoter (Harikrishna *et al*., 1996) in the pBluescript II phagemid cloning vector (Agilent Technologies, Santa Clara, CA, USA) was used as a PCR template for site-directed mutagenesis.

All PCRs were carried out using either *Taq* DNA polymerase (Promega Corporation, Madison, WI, USA) or Phusion High-Fidelity DNA polymerase (New England Biolabs, Ipswitch, MA, USA), according to the manufacturers' instructions.

To create the construct with an additional N-glycosylation site at the HVa region of the S_{11}-RNase (hereafter identified as $Glc^{C2/HVa}$), the AAA codon for lysine in the sequence KLTYNYFSD was changed into a AAT codon for asparagine (K72N) using mutagenic primers Glc^{C2} A (5'-AAGTACTGCAAGCCAAATCTTACCTATAACTAT-3') and Glc^{C2} B (5'-ATAGTTATAGGTAAGATTTGGCTTGCAGTACTT-3') and a PCR mutagenesis kit (Agilent Technologies Santa Clara, CA, USA) according to the manufacturer's instructions.

This latter construction $Glc^{C2/HVa}$ was further modified to remove the conserved site in C2, by again changing the AAC

codon at position 50 to a CAA codon (N50Q). Such a modified S_{11}-RNase was identified as Glc^{HVa}. Here the PCR overlap mutagenic primers were $Glc^{C2/HVa}$ A (5'-CGAATAGTTCCA AAACAATTTACGATTCACGG-3') and $Glc^{C2/HVa}$ B (5'-CCGTG AATCGTAAATTGTTTTGGAACTATTCG-3'), while the two flanking primers in the vector, forward-HindIII 5'- (5'-GCGGCG CGTTCAAGCTTTCTAGAAGATCTCT-3') and reverse-EcoR-1stop (5'-CTCTGAATTCAAGGACATACATTTGATAG-3') contained endonuclease restriction sites to facilitate further cloning steps. Both $Glc^{C2/HVa}$ and S_{11}-Glc^{HVa} constructs were ligated into pBluescript II using restriction endonucleases *Hin*dIII and *Eco*RI (Promega), and subcloned into the pBIN19 binary plant transformation vector (Clontech, Palo Alto, CA, USA) using the same enzymes.

To create the construct lacking the original glycosylation motif in the C2 region (hereafter identified as *NoGlc*), site-directed mutagenesis by PCR overlap extension (Higuchi *et al*., 1988) was used to change the AAC codon for asparagine at position 50 of S_{11}-RNase to a CAA codon for glutamine (N50Q). Mutagenic primers *NoGlc* A (5'-CAACGAATAGTTCCAAAA CAATTTACGATTCACGGTC-3') and *NoGlc* B (5'-GACCGTGA ATCGTAAATTGTTTTGGAACTATTCGTTG-3') were used in combination with flanking primers T7 and T3, respectively, first to generate separate mutated overlapping PCR fragments. These fragments were purified and mixed, and then used with primers T7 and T3 to generate a full-length *NoGlc* fragment. That fragment was digested using restriction endonucleases *Xba*I and *Sal*I (Promega), cloned into pBluescript II, then subcloned into the pBIN19 binary plant transformation vector (Clontech) using the same enzymes.

Sequencing validated all three mutated ChiP-S_{11}-RNase constructs after cloning into the pBIN19 transformation vector (Clontech), which were then transformed into *Agrobacterium tumefaciens* LBA4404. Plants were transformed by the leaf disc method and regenerated as described previously (Matton *et al*., 1997) using the *S. chacoense* genotype G4 carrying the $S_{12}S_{14}$ alleles.

Crosses and pollen observation

Genetic crosses of transgenic plants were performed using pollen freshly collected from various genotypes as described (Qin *et al*., 2001). Where appropriate, styles were stained with aniline blue 48 h post-pollination and examined by fluorescence microscopy to assess pollen tube growth (Matton *et al*., 1997).

Western blot analyses and deglycosylation of S-RNases

Stylar proteins were extracted from both transgenic and non-transgenic plants as described (Qin *et al*., 2001, 2005). The protein concentration of crude extracts from 10 styles of each plant was determined by Bradford protein assay (Bradford, 1976). Deglycosylated protein samples containing 40 µg of total proteins were prepared by digestion with peptide-N-glycosidase F (PNGase F) (New England Biolabs) according to the manufacturer's instructions. Non-deglycosylated samples were prepared by the same procedure but omitting the PNGase F enzyme.

Samples were analysed by SDS–PAGE with 20 µg of total stylar protein loaded per lane. Proteins were then transferred to Hybond C-Extra nitrocellulose membranes (GE Healthcare, Piscataway, NJ, USA) using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. Protein transfer was visualized after staining the membranes with Ponceau S using an ImageQuant LAS 4000 imaging system (GE Healthcare).

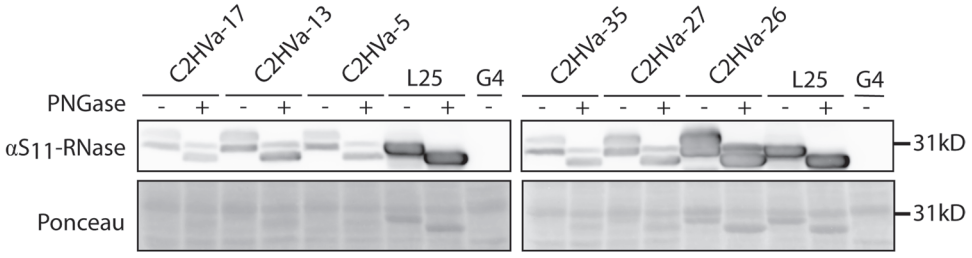
Immunoblots were performed as described (Qin *et al*., 2005) with a specific primary antibody raised against a 15 amino acid peptide corresponding to the HVa region of the S_{11}-RNase and a commercial horseradish peroxidase (HRP)-conjugated secondary antibody (Sigma-Aldrich Corporation, St Louis, MO, USA). HRP activity was visualized using the Immobilon Western Chemiluminescent HRP substrate kit (EMD Millipore Corporation, Billerica, MA, USA) according to the manufacturer's instructions and recorded using an ImageQuant LAS 4000 imaging system.

Results

$Glc^{C2/HVa}$ transgenic plants

A previous report of self-compatible *Nicotiana sylvestris* found that styles expressed high levels of a stylar 'relic S-RNase' (Golz *et al*., 1998). To explain why this species is self-compatible, the authors suggested that an N-glycosylation site inside the hypervariable HVa region might be involved. To test experimentally the hypothesis that a bulky polysaccharide moiety in the HVa region might prevent the pollen *S*-gene product from interacting with the S-RNase thus leading to compatibility, an S_{11}-RNase with an extra glycosylation site in the HVa region was first produced.

Protein levels corresponding to the $Glc^{C2/HVa}$ S-RNase, assessed by western blots, varied from almost undetectable to levels higher than wild-type S_{11}-RNases (Fig. 1). Genetic crosses assessed the impact of these variable levels of $Glc^{C2/HVa}$ on the incompatibility phenotype. All plants were self-incompatible and fully incompatible with pollen from the untransformed G4 ($S_{12}S_{14}$) line. In addition, crosses using pollen from PI 230582 ($S_{13}S_{14}$) were compatible as expected, due to the presence of compatible S_{13} pollen. The crosses with pollen from line L25 ($S_{11}S_{12}$) revealed that transgenic plants with low expression levels, such as line 17, had an incomplete S_{11}-rejection phenotype, whereas plants with higher expression levels (lines 5,13 26, 27, and 35) fully rejected S_{11} pollen (Fig. 1). Microscopic observations confirmed the complete arrest of S_{11} pollen tubes inside the styles of plants highly expressing the transgene. The rejection of S_{11} pollen was surprising, as it had been predicted that the glycosylation site in the HVa region, previously identified as one of the two allelic recognition domains in S_{11}-RNase (Matton *et al*., 1997), would block binding to this region of the S-RNase. To assess the possibility that the HVa site was not fully occupied, the stylar extracts were analysed before and after treatment with PNGase. Before treatment, two size classes of protein reacting with the anti-S_{11} antibody could be detected. One size class, more abundant and having the same apparent molecular weight as the authentic S_{11}-RNase (26 kDa), was interpreted as a monoglycosylated form of the protein. The second size class, with a slightly slower mobility (28 kDa), was interpreted as the diglycosylated form of the protein. This suggests that one of the two sites was indeed not fully occupied. Interestingly, two size classes of protein reacting with the anti-S_{11} antibody were still present after treatments with the PNGase. One of the bands had an apparent mol. wt of 24 kDa, corresponding to the fully deglycosylated S_{11}-RNase, while the other had a size commensurate with the monoglycosylated form which was interpreted as a sugar moiety resistant to PNGase. It is also noted that the same amount of antibody-reactive protein is detected in the sample before and after deglycosylation with PNGase, suggesting that the presence of the sugar group did not interfere with the antibody reaction. It is concluded that incomplete glycosylation of the



Pollen	Fruits/Flowers pollinated							
$S_{11}S_{12}$	7/12	0/13	0/12	0/6	5/5	0/11	0/12	0/12
$S_{13}S_{14}$	8/8	10/10	6/6	6/6	6/6	8/8	10/10	10/10

Fig. 1. Plants expressing a diglycosylated S_{11}-RNase have a normal S-phenotype. Glc^{C2HVa} plant lines express an S_{11}-RNase that contains a glycosylation site in the HVa region in addition to the normal and conserved glycosylation site in the C2 region. Western blot analysis of six selected plants shows a range of protein levels detectable using an anti-S_{11}-RNase antibody. The size of the bands in the absence of PNGase (–) is consistant with a mono- and a diglycosylated form, while in the presence of PNGase (+) the sizes are consistent with a non-glycosylated and a monoglycosylated form. L25 plants ($S_{11}S_{12}$) and the untransformed host G4 ($S_{12}S_{14}$) are shown as positive and negative controls, respectively. The pollination phenotype of each plant is shown for a representative pollination with $S_{11}S_{12}$ and $S_{13}S_{14}$ tester stocks and is reported as the number of fruits set per pollination.

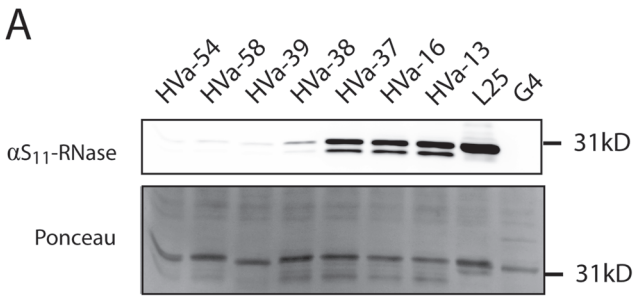
HVa glycosylation site could result in an S_{11} pollen rejection phenotype due to an S_{11}-RNase glycosylated only at the C2 region, as this monoglycosylated form is expected to be identical to the wild type.

Glc^{HVa} transgenic plants

The inability of the $Glc^{C2/HVa}$ plants to glycosylate both sites fully may have resulted from the proximity of the two sites and a difficulty in accessing sufficient amounts of the glycosylating precursor. Another modified S_{11}-RNase, termed Glc^{HVa}, which contains only a single glycosylation site inside the HVa region was therefore produced. The genetic analysis of 41 Glc^{HVa} transgenics revealed that all plants were self-incompatible and incompatible with line G4 ($S_{12}S_{14}$). When crossed with pollen from line L25 ($S_{11}S_{12}$), three individuals termed lines Glc^{HVa}-13, -16, and -37, were found to reject S_{11} pollen completely (Fig. 2A). This pollen rejection phenotype correlated with the amounts of the Glc^{HVa}-RNase which was similar to that found in the L25 control plants as detected by western blot analysis (Fig. 2A). Pollen tubes in their styles displayed the reaction characteristic of an SI response when examined by fluorescence microscopy. Most of the pollen tubes were arrested at mid-style, although in rare instances a very small number of pollen tubes were found near the stylar basis but none entering the ovarian region.

Surprisingly, however, when these three individuals were crossed with PI 230582 ($S_{13}S_{14}$), they were found to reject S_{13} pollen partially, which was expected to be fully compatible with the modified S_{11}-RNase (Fig. 2A). Western blots revealed the presence of two size classes of protein reacting with the anti-S_{11} antibody (Fig. 2A). Analysis of the extracts from these three individuals before and after treatment with the PNGase were therefore undertaken (Fig. 2B). In untreated extracts, the upper band (31 kDa) was systematically more intense than the lower band, whereas after treatment with the PNGase, the lower band appeared more intense. These samples also show the same amount of signal before and after deglycosylation, again suggesting that the sugar group in the HVa region does not interfere with the antibody reaction. The lower band in the three transgenics corresponds to the unglycosylated form, as its size is identical to the enzymatically deglycosylated L25 S_{11}-RNase. The upper band in the three transgenic lines thus corresponds to the monoglycosylated form, and this band is more intense than the lower band, indicating an increase in glycosylation at the HV site compared with the double glycosylated $Glc^{C2/HVa}$ (Fig. 2B).

Two possibilities could account for the acquisition of this unexpected dual-specific pollen rejection phenotype. In the first, one of the two forms of the protein could have acquired the dual-specific phenotype, perhaps due to the modified amino acid in the HVa region similar to a dual-specific allele previously generated by site-directed mutagenesis in the HVa region of the S_{11}-RNase (Table 1) (Matton *et al*., 1999). Alternatively, as two S-RNase forms (glycosylated and non-glycosylated) are found in the transgenics, one form might confer the S_{11}-rejection phenotype while the other might confer the S_{13}-rejection phenotype. To assess the possibility that a single form of the Glc^{HVa}-RNase could recognize and reject both S_{11} and S_{13} pollen, these individuals were submitted to the heteroallelic pollen test (Luu *et al*., 2001) in which the previously described dual-specific allele was found to reject the normally compatible heteroallelic diploid pollen derived from a $S_{11}S_{11}$ $S_{13}S_{13}$ individual (Luu *et al*., 2001). As shown (Fig. 2A), this behaviour is also evident in Glc^{HVa}-RNase-expressing plants, supporting its identification as a new dual-specific allele.



Pollen	Fruits/Flowers pollinated								
S11S12	8/8	7/7	7/7	6/8	0/16	0/13	0/15	0/6	5/5
S13S14	6/6	6/6	7/7	6/6	4/14	5/13	8/16	5/5	6/6
S15S16	7/7	8/8	6/6	6/6	7/7	7/7	5/5	6/7	5/5
S11S11S13S13	7/7	8/9	6/6	7/7	2/10	2/12	3/11	6/7	5/5

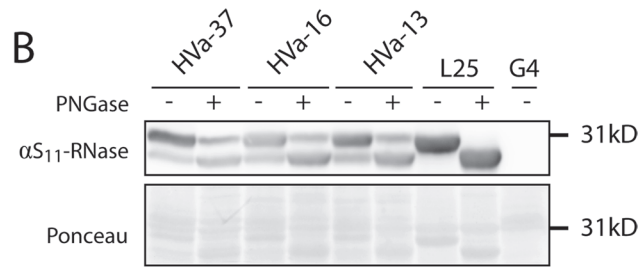


Fig. 2. Plants expressing the Glc^{HVa}-RNase have an unusual dual specificity incompatibility phenotype. Western blot analysis of seven plant lines expressing an S_{11}-RNase engineered to contain only a single glycosylation site in the HVa region show a wide range of protein levels (A). L25 plants ($S_{11}S_{12}$) and the untransformed host G4 ($S_{12}S_{14}$) are shown as positive and negative controls, respectively, and the pollination phenotype of each plant is shown for a representative pollination with $S_{11}S_{12}$, $S_{13}S_{14}$, $S_{15}S_{16}$, and $S_{11}S_{11}S_{31}S_{13}$ tester stocks. The three highly expressing lines are shown with (+) and without (–) PNGase treatment (B).

NoGlc transgenic plants

To test the possibility that a non-glycosylated S-RNase would represent a universal rejector, an additional construct, called NoGlc, lacking the original glycosylation motif in the C2 region was generated. The genetic analysis of 81 NoGlc transgenics revealed that all plants were self-incompatible and incompatible with line G4. Western blot analyses performed on these plants showed a variable level of signal. The crosses with pollen from line L25 ($S_{11}S_{12}$) revealed that transgenic plants with low expression levels had an incomplete S_{11}-rejection phenotype, whereas plants with higher expression

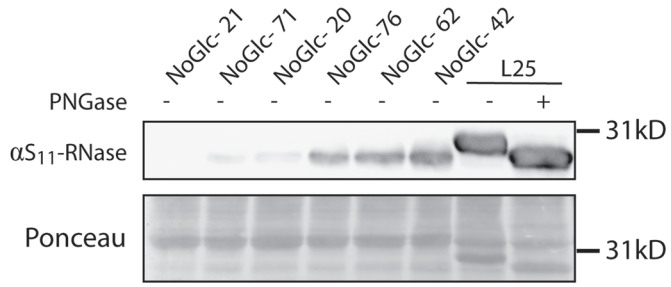
Table 1. Sequence comparison of the Glc^{HVa} and the previously described dual-specific HVapb-RNase.

S-RNase	HVa sequence	HVb sequence
S_{11}-RNase	KPKLTYNYF	IDQASARK
Glc^{HVa}	KPNLTYNYF	IDQASARK
HVapb	KPKLNYNFF	IDQASALK
S_{13}-RNase	KPKLNYKFF	IDQASALK

levels fully rejected S_{11} pollen (Fig. 3). In order to assess whether or not the plants highly expressing the transgene were able to reject other pollen types, they were pollinated with pollen from line PI 230582 ($S_{13}S_{14}$). All crosses resulted in full compatibility. These plants were also crossed with pollen from an unrelated genotype PI458312 ($S_{15}S_{16}$). The results of the crosses indicated once again full compatibility (Fig. 3). From these results, it is concluded that the lack of glycosylation of the S-RNases does not prevent them from being functional and does not generate an S-RNase resistant to degradation.

Discussion

In this study, two aspects of S-RNase glycosylation were assessed. It was first sought to determine if the presence of an N-linked glycan inside the HVa region would affect pollen



Pollen	Fruits/Flowers pollinated						
S11S12	16/16	8/19	3/13	0/15	0/19	0/17	0/7
S13S14	7/7	4/4	6/6	4/4	6/6	7/7	7/7
S15S16	5/5	5/5	6/6	5/5	7/7	7/7	6/6

Fig. 3. Plants expressing the NoGlc S-RNase have a normal S-phenotype. Western blot analysis of seven plant lines expressing an S-RNase lacking all glycosylation sites shows a range of protein levels. L25 plants are shown as a positive control, and the pollination phenotype of each plant is shown for a representative pollination with $S_{11}S_{12}$, $S_{13}S_{14}$, and $S_{15}S_{16}$ tester stocks.

recognition, as suggested by a study using the self-compatible *N. sylvestris* expressing a stylar 'relic S-RNase' with an N-glycosylation site inside the HVa region (Golz *et al*., 1998). Secondly, it was thought to be of interest to determine if a non-glycosylated S-RNase could reject all different pollen types as proposed by Oxley *et al*. (1996, 1998).

Relic S-RNases, found in both self-compatible and self-incompatible species (Lee *et al*., 1992; Golz *et al*., 1998; Liang *et al*., 2003; Roldan *et al*., 2010), are a particular type of S-like RNases, structurally similar to functional S-RNases and expressed exclusively in the pistil (Roldan *et al*., 2010). However, in common with S-like RNases, they are not coded by the *S*-locus and are not associated with SI. The relic S-RNase from *N. sylvestris* displayed levels of S-RNase activity comparable with those found in self-incompatible *Nicotiana* species (Golz *et al*., 1998), and it was suggested that the bulky polysaccharide moiety in the HVa region might prevent the pollen *S*-gene product from interacting with the S-RNase, leading to compatibility.

The explanation proposed by Golz *et al*. (1998) predicts that blocking recognition of the HVa region would lead to compatibility, even in styles with high levels of transgene expression. This is an interesting prediction since a new model of the pollen rejection mechanism, termed the collaborative non-self-recognition system (Kubo *et al*., 2010; Kakui *et al*., 2011), actually predicts the opposite result. The collaborative non-self-recognition model, based on the finding that the S-locus contains a number of pollen recognition F-box proteins, proposes that one or a combination of these F-box proteins is able to recognize and degrade any S-RNase with the exception of that encoded by the same *S*-locus. Blocking access to the recognition domain in the HVa region should thus make the S-RNase resistant to degradation, resulting in pollen rejection.

The first attempt to introduce a glycosylation site in the HVa region, the $Glc^{HVa/C2}$ plants, produced five individuals that turned out to reject S_{11} pollen completely while allowing passage of S_{13} pollen. This S-RNase is not a universal rejector. However, an analysis of the S-RNase by western blot revealed that glycosylation at the site in the HVa region was incomplete. This conclusion is based on the finding that the $Glc^{C2/HVa}$ plants express two different forms of the S-RNase with distinct molecular weights (Fig. 1). These two size classes are interpreted as mono- and diglycosylated forms of the enzyme, with the monoglycosylated form being more abundant. The monoglycosylated form is also interpreted as equivalent to the wild-type S_{11}-RNase (i.e. glycosylated at the site in the C2 region), as the wild-type S_{11}-RNase is always fully glycosylated at the C2 site (Fig. 1) and the HVa glycosylation site only becomes available to the glycosylation machinery after the C2 region has entered the endoplasmic reticulum.

To attempt to overcome the problem of incomplete glycosylation in the HVa region, the Glc^{HVa} S-RNase, whose single glycosylation site was moved from the C2 region to the HVa domain, was constructed. Unfortunately, this protein also turned out to be incompletely glycosylated, as two bands can be observed by gel analysis (Fig. 2). It is possible that the protein context surrounding the HVa site is not as efficient as that surrounding the C2 site, which is always fully glycosylated. In addition, since two bands are observed even after a treatment with the PNGase (Fig. 2B), it seems likely that the protein context surrounding the HVa region may also allow heterogeneity in the sugar groups themselves. This is suggested by the observation that the ratio of signal intensity between the upper (monoglycosylated) and lower (deglycosylated) forms is significantly different before and after PNGase treatment. This was interpreted to mean that part of the monoglycosylated form is sensitive, and part resistant, to PNGase activity. This is not infrequent in plants, as addition of α(1–3)-fucose to the *N*-acetyl glucosamine attached to the asparagine protects the sugar group against PNGase treatment (Tretter *et al*., 1991).

Even more surprisingly, however, the three plants that contained sufficient S-RNase to reject S_{11} pollen were also able to reject S_{13} pollen partially. At least in part, this double specificity may relate to the inherent similarity between S_{11}- and S_{13}-RNases as the two mature protein sequences share 95% sequence identity. Indeed, four of the amino acid differences between the two lie in the HV region, and it was previously reported that mutation of three of these in the S_{11} sequence also allowed rejection of both S_{11} and S_{13} pollen (Matton *et al*., 1999). However, the C2 region of the previously generated dual-specific *S*-allele is fully glycosylated and thus differs from the Glc^{HVa} plants in which mutagenesis of the acceptor site in the C2 region blocks glycosylation at this site.

It is proposed that rejection of both S_{11} pollen and S_{13} pollen occurs because the unglycosylated form of the RNase in transgenic Glc^{HVa} plants has acquired a dual specificity incompatibility phenotype. This phenotype could be explained by the self-recognition model (Fig. 4) if the site used to recognize the RNase as an S_{13} haplotype was partially occluded by the sugar group in the C2 region, thus explaining why the $Glc^{C2/HVa}$ cannot reject S_{13} pollen. This idea is supported by the observation that in the Rosaceae S-RNases can be phenotypically different despite having identical sequences in the HV region (Wunsch and Hormaza, 2004; Zisovich *et al*., 2004). Interestingly, this observation also implies that sugar groups remain on the S-RNase even after entry into the pollen tube cytoplasm.

However, it is important to note that the dual-specific phenotype can also be accommodated by the collaborative non-self-recognition model. In this case, since the incompatibility of an individual haplotype results from the lack of a specific SLF capable of targeting the S-RNase for degradation, glycosylation of the Glc^{HVa} RNase would impede binding by the SLF that normally recognizes and degrades the S_{13}-RNase.

The surprising rejection of S_{13} pollen, which occurs at least partially in three of the Glc^{HVa} plants, is reminiscent of a previously characterized dual-specific form of the S_{11}-RNase (Matton *et al*., 1999). The new dual-specific allele shares with the previously described dual-specific allele a modification in the HV region (Table 1) that replaces a positively charged amino acid (lysine in the HVa region of the Glc^{HVa}, and arginine in the HVb region of the HVapb) with an uncharged amino acid (asparagine and leucine for Glc^{HVa} and HVapb,

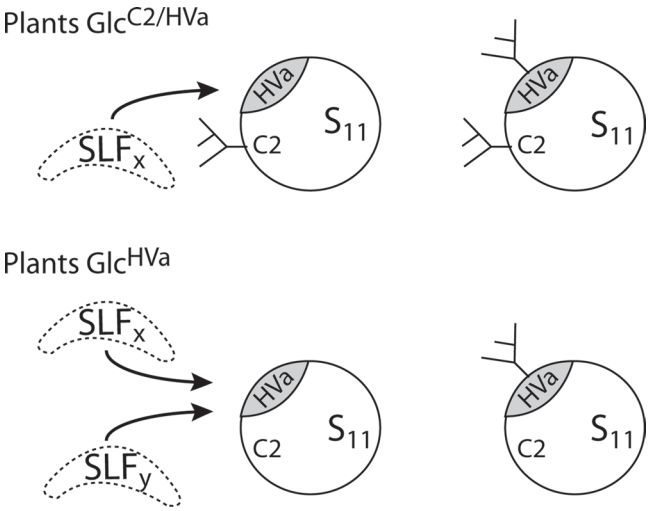


Fig. 4. Model for the different S-RNases produced by Glc$^{C2/HVa}$ and Glc^{HVa} transgenic lines. Each of the transgenic lines produces two S-RNase forms (spheres), differing from one another with regard to glycosylation at the newly introduced site in the HVa region. The glycan groups are shown as twigs at the protein surface, in either the hypervariable HVa region or the conserved C2 region. A hypothetical interaction with the pollen component (dotted line) is shown for recognition by either the S_{11} or S_{13} pollen (SLFx and SLFy, respectively).

respectively). Curiously, the new allele differs from the previous allele in that the dual specificity is only revealed when the S-RNase cannot be glycosylated in the normal C2 region. It is important to stress that the unusual rejection phenotype of the Glc^{HVa} plants that was observed here is not due to overexpression of the transgene, as the S-RNase levels measured in the styles are lower than those found for wild-type plants (Fig. 2). It is also not due to an ability to reject any pollen type, as pollen from an $S_{15}S_{16}$ stock is accepted. Furthermore, it is unlikely to result from fortuitous insertion of the transgene in a component of the SI machinery required for allowing pollen to resist the effects of elevated S-RNase activity, as the three transgenic lines with this same phenotype represent independent insertion events. Lastly, the phenotype is not due to female sterility, as all lines set seeded fruits after compatible pollinations.

It is also of interest to note that the NoGlc plants have definitely laid to rest the 'universal rejector' hypothesis. This model posits the glycan moiety to be a key element involved in recognition of all non-self S-RNases. For example, compatible pollinations would exploit recognition of the conserved sugar moiety in order to target all S-RNases to the proteasome, while during incompatible pollinations, interaction between the C2-glycan and the proteasomal targeting system would be blocked by allele-specific binding to the recognition domain in the HV region. This is clearly not the case, as NoGlc plants do not reject all pollen but behave as though they expressed a normal S_{11}-RNase. In particular, $S_{15}S_{16}$ pollen is able to set fruit. These results firmly rule out the possibility that the C2 glycosylation site might be an important recognition site for the degradation of the S-RNase by providing a universal mechanism for detoxifying S-RNases. However, it is noted that the sugar group in the C2 region can in some cases affect the pollen rejection phenotype of the S-RNase, as the presence of the glycan moiety at the C2 position appears to interfere with recognition of the Glc$^{C2/HVa}$ RNase as an S_{13}-RNase.

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